Type II collagen is a target antigen of clonally expanded T cells in the synovium of patients with rheumatoid arthritis

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Abstract

***Objective*—To investigate whether type II collagen (CII) is recognised by oligoclonally expanded synovial T cells of patients with rheumatoid arthritis (RA).**

***Methods*—Peripheral blood mononuclear cells (PBMC) from 15 RA patients were stimulated with CII in vitro. T cell clones expanded by such stimulation were compared with the clonally expanded synovial T cells by using T cell receptor (TCR) B chain gene specific reverse transcription-polymerase chain reaction and subsequent single strand conformation polymorphism analyses.**

***Results*—Stimulation of the heterogeneous peripheral T cells with CII induced clonal expansion of T cells. In three of 15 patients, a proportion of these clones (approximately 17% to 25%) was found to be identical to expanded T cell clones in the synovium in vivo.**

***Conclusion*—T cell clones that had TCR CDR3 sequences identical to those induced by purified CII were found in a proportion of RA patients. This finding suggests that CII is recognised by T cells that accumulate clonally in RA joints. Oligoclonal T cell expansion in RA joints is probably driven, at least in part, by intra-articular components such as CII.**

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Rheumatoid arthritis (RA) is characterised by chronic inflammation of the synovium with subsequent joint destruction. Although the pathogenesis of RA is not fully understood, there is some evidence to suggest the involvement of T cells in the inflammatory process. Firstly, marked infiltration of T cells is present in the affected joints of RA and most of these cells are CD4+CD45RO+ (activated and memory type) T cells.[1] Secondly, susceptibility to RA is associated with selected HLA haplotypes such as HLA-DR4.[2] Thirdly, transfer of T cells induces RA-like synovitis in experimental animal models of RA.[3] Furthermore, previous studies including those from our laboratory[4 5] have demonstrated that the synovium infiltrating T cells expand oligoclonally, suggesting an antigen driven immune reaction.

Several intra-articular components, such as type II collagen (CII) and proteoglycan, are considered as potential RA specific autoantigens.[6 7] In particular, CII responding T cell lines or clones have been established in vitro from synovial T cells of RA.[6] To establish the role of these antigens in RA, previous studies have reported the frequent presence of anti-CII antibodies in RA patients[8 9] and that oral administration of CII to RA patients results in amelioration of symptoms of RA.[10]

In this study, we analysed the T cell receptor (TCR) B gene to investigate whether CII was actually recognised by the clonally expanded T cells in the RA joints. Specifically, we stimulated peripheral blood mononuclear cell (PBMC) obtained from RA patients with CII and then compared the resultant clones with the in vivo accumulated ones in the joints of the same patient. For this purpose, we used a combination of reverse transcription-polymerase chain reaction (RT-PCR) amplification of the TCR complementarity determining region (CDR3) gene and the subsequent separation by single strand conformation polymorphism (SSCP).[4 5 11] The results showed that a proportion of T cell clones in PBMC induced by stimulation with purified CII was identical to those of in vivo synovial T cells, suggesting that synovial T cell clonotypes proliferate clonally, recognising CII in the RA joints.

Methods

PATIENTS

Fifteen Japanese patients with RA (14 women and one man), diagnosed according to the revised criteria of the American College of Rheumatology,[12] were enrolled in this study. Table 1 shows the patient data.

CLINICAL SAMPLES

All clinical samples were obtained with informed consent. Using heparinised blood or synovial fluid sample from each patient, mononuclear cells were separated by a standard density gradient centrifugation. Synovial tissues, obtained by synovectomy, were minced into small pieces and then immediately immersed in a denaturing guanidine solution for RNA preparation.

CELL CULTURE

PBMC (2×10^6) were suspended in RPMI-1640 culture medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10% heat inactivated fetal calf serum, and a suboptimal concentration (2 IU/ml) of recombinant human interleukin (IL) 2 (rIL2, Shionogi and Company, Osaka, Japan). The cells were cultured for seven days

in 48 well flat bottom plates with or without 25 μg/ml of human type II collagen (hCII, Chemicon International Inc, Temecula, CA) or bovine type II collagen (bCII, Elastin Products Company, Inc, Owensville, MO), or purified protein derivative (PPD) (10 μg/ml; Japan BCG Laboratory, Tokyo, Japan). The cultured cells were subsequently washed, immersed in a denaturing guanidine solution, and processed for RNA extraction.

SEPARATION OF TCR B GENES BY THE SINGLE STRAND CONFORMATION POLYMORPHISM AFTER AMPLIFICATION BY RT-PCR (RT-PCR-SSCP)

RT-PCR-SSCP analysis was performed as reported previously[4 5 11] with minor modifications. In brief, RNA, extracted from each sample was converted to cDNA by reaction with reverse transcriptase (1 U, Gibco BRL, Gaithersburg, MD) and random hexamer oligonucleotides (100 pmol, Gibco BRL) at 42°C for three hours. For amplification of TCR B genes, we used 100 ng of cDNA in 25 μl of a solution containing 200 nM dNTP, 0.5 IU Taq DNA polymerase (Boehringer Mannheim GmbH, Mannheim, Germany) and 50 pmol each of a common BC primer and one of 22 BV primers.[11] Amplification of DNA was performed using 36 cycles of 94°C for 1.5 minutes, 58°C for two minutes, 72°C for two minutes. The amplified DNA was diluted, heat denatured and electrophoresed in non-denaturing 4% polyacrylamide gel containing 10% glycerol. After transfer to a nylon membrane (Gene Screen, Biotechnology Systems, NEN Research Products, Boston, MA), the separated DNA was hybridised with a biotinylated internal TCR BC probe.[11] Finally, the bound probe was visualised by Phototope detection kit (Bio-Rad, Hercules, CA).

NUCLEOTIDE SEQUENCING

TCR B genes, either amplified by the first PCR or extracted from SSCP gels, were re-amplified using a TCR BV primer with an EcoRI recognising sequence and a common TCR BC primer with a HindIII site. The re-amplified TCR B genes were subcloned to a plasmid vector (pBluescript II, Toyobo, Osaka). Nucleotide sequences of the TCR B genes were determined by the di-deoxy method using 377 DNA Sequencing System (Perkin Elmer/Applied Biosystems, Foster, CA).

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETECTION OF ANTI-CII ANTIBODY

The ELISA was performed as previously reported.[8] In brief, a 96 well flat bottom plate (Becton Dickinson, Williams Drive Oxnard, CA) was coated with 100 μl of hCII (10 μg/ml phosphate buffered saline (PBS), pH 7.4, Chemicon) at 4°C overnight. After blocking with 1% skimmed milk/0.05% Tween-PBS at room temperature for one hour, the plate was washed four times, and 100 μl of serum (diluted at × 100) was added to each well, and incubated for one hour at room temperature. After repeated washing, 100 μl of horse radish peroxidase (HRP) conjugated protein G (ZYMED Laboratories Inc, South San Francisco, CA), which was diluted at × 2000, was added and the plate was further incubated for one hour. After repeated washing, enzymatic activity of peroxidase bound to protein G was measured using a microplate reader (Bio-Rad, Hercules, CA). The experiments were performed as triplicate. OD value was calculated by the following formula:

OD value = wells coated with hCII−wells without hCII

A serum was identified as positive if its OD value was higher than the mean +3 SD of results of serum samples from randomly selected healthy volunteers.

Results

COMPARISON OF TCR B GENE CLONOTYPES BETWEEN CII STIMULATED PBMC AND SYNOVIAL T CELLS BY RT-PCR-SSCP

Firstly, we investigated whether CII was a target antigen of expanded T cell clones in the synovium of RA patients. Specifically, we stimulated PBMC with CII in vitro to expand CII specific T cell clones from the circulating population. In the next step, T cell clonotypes expanded against CII were detected by the RT-PCR-SSCP analysis then compared with

Table 1 Patient profiles and the number of the CII recognised T cells in the synovium*

Patient	*Sex*	*Age*	*Disease duration (y)*	*Stage class*		*ESR (mm 1st h)*	*CRP (mg/dl)*	*RF (U/ml)*	*Sample†*	*HLA-DR B1* loci*	*hCII‡*	*bCII§*	*Anti-CII antibody*
1	female	71	4	4	3	32	4.9	10.3	LK	01, 08	2	NT	—
2	female	45	12	4	2	22	<0.5	<2	Bil K	0405, 1405	1	1	—
3	female	77	8	3	2	33	2.8	38	LK	1501, 1502	1	—	—
4	female	54	4	3	3	78	2.0	349	LK	0405, 1201	—	—	NT
5	female	65	10	4	2	49	0.8	24	LK (SF)	0901, —	—	—	—
6	female	69	6	4	2	70	10.6	53	Bil K	0405, 0901	—	—	—
7	female	53	7	4	2	5	<0.5	6	LK (SF)	01, 1602	—	—	NT
8	female	62	7	4	2	66	4.3	9	LK (SF)	0803, 1501	—	—	NT
9	female	60	7	4	2	78	5.8	160	RK	0405, 1502	—	—	NT
10	female	66	8	3	3	17	<0.5	<2	LK	0405, 1201	—	—	NT
11	female	60	17	4	3	30	5.4	139	RK	0405, 1302	—	NT	NT
12	female	77	10	3	3	30	<0.5	20	LK	1501, 1602	—	NT	—
13	female	74	10	3	2	76	4.8	27	LK	0901, 1406	—	NT	NT
14	female	60	25	3	3	98	1	159	Bil K	1302, 1502	—	NT	NT
15	male	75	5	1	2	78	4.7	101	RK (SF)	0405, 0901	—	—	NT

*Average of age and disease duration in patients are 64.5 (9.5) or 9.3 (5.5) years (mean (SD)), respectively.
†LK, RK, and Bil K indicate left knee, right knee and bilateral knees, respectively. Samples noted as SF indicates T cells derived from synovial fluid; otherwise synovial membrane was used as described in Methods.
‡The number of the identical T cell clones between hCII stimulated T cell and in vivo expanded synovial T cell clones.
§The number of the identical T cell clones between bCII stimulated T cell and in vivo expanded synovial T cell clones.
NT = not tested.

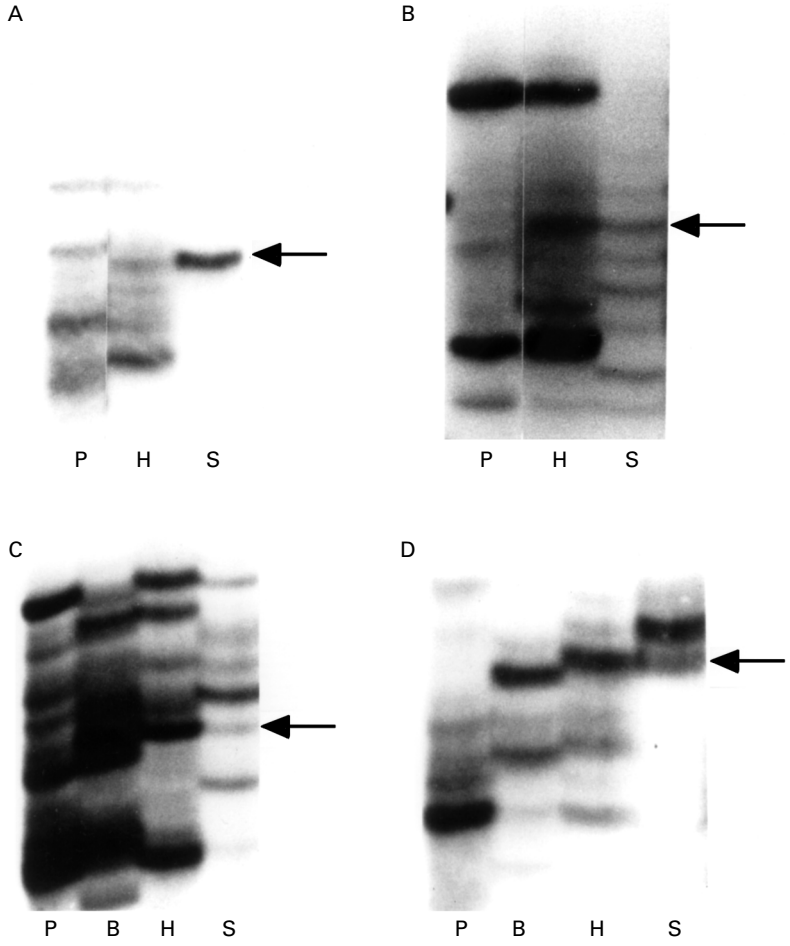

Figure 1 Comparison of T cell clonotypes between CII stimulated peripheral T cells and in vivo synovial T cells by RT-PCR-SSCP. TCR BV gene products were electrophoresed on the same SSCP gels. (A) BV13S1 from patient 1; (B), BV12 from patient 1; (C), BV1 from patient 2, (D), BV16 from patient 3. P = PBMC cultured with rIL2 alone; B = PBMC cultured with bCII and rIL2; H = PBMC cultured with hCII and rIL2; S = in vivo synovial T cells. Arrows indicate bands with identical migration between hCII stimulated PBMC (lane H) and synovial tissue infiltrating T cells in vivo (lane S).

the in vivo expanded clones in the RA affected joint. We also analysed T cell clonality of unstimulated PBMC and of synovial tissue by RT-PCR-SSCP. PBMC produced smear-like broad bands mostly on the SSCP gels, which indicated a heterogeneous TCR BV repertoire (data not shown). In contrast, synovium infiltrating T cells showed several distinct bands on the gels in most of the tested TCR BV families, indicating oligoclonal T cell expansion (data not shown).

Next, we cultured PBMC in vitro with a suboptimal dose of rIL2 with or without hCII, and then analysed the T cell clonotypes in a manner similar to that described above. The results showed that stimulation with hCII and rIL2, or with rIL2 alone, induced several new bands on the SSCP gels—that is, clonal expansion of T cells in the stimulated population. The generated T cell clones were compared with in vivo expanded clones of each RA patient. Characteristically, identical clones were detected as distinct bands with identical migration on the same SSCP gel. As a result, four T cell clonotypes were generated by stimulation with hCII and rIL2, but not with rIL2 alone and were found to be identical to synovial T cell clonotypes (a clone in BV13S1 T cells and one in BV 12 in patient 1; a clone in BV1 in patient 2; one in BV16 in patient 3; fig 1A–D, arrows, and table 1). Each of these clones were among four, four, six and four clones that were induced by hCII stimulations, respectively, thus indicating the ratio as 16.7% (1 of 6) to 25% (1 of 4) of all of the induced expanding clones within each BV family. Conversely, the ratio of these identical clones among in vivo expanding clones in the synovium were: patient 1, BV13S1: 1 of 1=100%, patient 1, BV12: 1 of 5=20%, patient 2, BV1: 1 of 6=16.7%, and patient 3, BV16: 1 of 2=50%. As the clones that were detected in hCII stimulated cells were likely to have recognised and responded to hCII, the expanded T cell clones that carried the same TCR gene in the synovium (fig 1A–D lane S) were also considered to recognise hCII. Thus, the ratio of the T cell clones, which had identical TCR CDR3 sequence to those induced by stimulation with purified CII and were expanding in the synovium, were estimated to be approximately 16.7–100%. Note that such possibly "CII recognising" T cell clones were detected only in three of 15 patients tested (table 1).

We also tested in 10 of the 15 patients whether bCII stimulated T cells were identical to the clonally expanded T cells in the synovium. In patient 2, one of the bCII stimulated T cell clones was identical to clonally expanded T cells in the synovium (fig 1C, lane B). This T cell clone was also identical to the hCII stimulated T cell clone. However, other T cell clones induced by bCII stimulation were not identical to the expanded T cell clones in the synovium.

NUCLEOTIDE SEQUENCE ANALYSIS OF TCR B GENES OF CII RESPONSIVE T CELLS

Next, we confirmed the above finding quantitatively by random nucleotide sequencing. Specifically, we subcloned TCR BV13S1 gene products from patient 1 (fig 1A) into a plasmid vector, and determined the nucleotide sequences of the TCR B chain, including the CDR3, in more than 20 gene products that were randomly selected in each sample. Table 2A summarises the results. In the synovium, the TCR BV clonotype of BV13S1-SLG-BJ2S2 was dominant and its frequency was 70% of the BV13S1 gene family. PBMC cultured with a suboptimal dose of rIL2 alone also displayed seven clonally expanded T cell clonotypes. However, none of them was identical to the dominant clones in the synovium. On the other hand, it was shown that PBMC stimulated with hCII and a suboptimal dose of rIL2 contained the TCR BV13S1-SLG-BJ2S2 as one of the dominant TCR clonotypes (19%, table 2A). As a control antigen, PBMC was also stimulated with PPD and the sequence of TCR BV13S1 gene was analysed. The results showed clonal expansion of T cells, none of which were identical to whose induced by CII or those found in the synovium (table 2A). Taken together, these results suggested that the T cell clonotype with

Table 2 Amino acid sequence of CDR3 of TCR B chain

A Sequences selected at random from BV13S1 gene samples of patient 1. PBMC cultured with rIL2 alone

BV	*NDN*	*Bj*		*Frequency*	*(%)*
CASS	RLSG	SGANVLTFGA	(BJ2S6)	4/26	15
CAS	KVDSIQGA	QYFG	(BJ2S5)	3/26	12
CASS	SRD	GYTFGSGTRL	(BJ1S2)	2/26	8.0
CASS	EAYGGG	TDTQYFG	(BJ2S3)	2/26	8.0
CASS	VSWTGEPI	QYFG	(BJ2S4)	2/26	8.0
CASS	RHVEEG	YEQYFGP	(BJ2S7)	2/26	8.0
CAS	MASAGP	YEQYFGP	(BJ2S7)	2/26	8.0
CASS	ETGI	TEAFFGQGTR	(BJ1S1)	1/26	4.0
CASS	EAEG	NYGYTFGSGT	(BJ1S2)	1/26	4.0
CASS	EFGRGQD	QPQHFGD	(BJ1S5)	1/26	4.0
CAS	LMDRGPMG	QFFG	(BJ2S1)	1/26	4.0
CAS	RSQGAG	TDTQYFG	(BJ2S3)	1/26	4.0
CASSY	GSGTGVK	QYFG	(BJ2S7)	1/26	4.0
CASS	GRDRG	SYEQYFG	(BJ2S7)	1/26	4.0
CAS	GRV	YEQYFGPGCR	(BJ2S7)	1/26	4.0
CASS	EFWAGAG	EQYFGPG	(BJ2S7)	1/26	4.0

PBMC stimulated with human type II collagen

BV	*NDN*	*Bj*		*Frequency*	*(%)*
CAS	SLG	TGELFFGEG	(BJ2S2)	4/2	19
CASS	EGASG	YNEQFFG	(BJ2S1)	3/21	14
CAS	RDDRPP	TGELFFG	(BJ2S2)	3/21	14
CASSY	SSSSGR	TDTQYFG	(BJ2S3)	2/21	10
CASS	ESSWTSGK	AKWY	(BJ2S1)	1/21	5.0
CASS	EVGRRQ	QFFGPPT	(BJ2S1)	1/21	5.0
CASS	EAGHEY	TGELFFG	(BJ2S2)	1/21	5.0
CAS	RDDKAP	TGELFFG	(BJ2S2)	1/21	5.0
CAS	STGVG	TDTQYFG	(BJ2S3)	1/21	5.0
CASS	ERAGKD	YEQYFGP	(BJ2S7)	1/21	5.0
CASS	SERG	SYEQYFGPGT	(BJ2S7)	1/21	5.0
CASSY	YGGST	YEQYFGP	(BJ2S7)	1/21	5.0
CASSY	GALREA	YEQYFGP	(BJ2S7)	1/21	5.0

PBMC stimulated with purified protein derivative (PPD)

BV	*NDN*	*Bj*		*Frequency*	*(%)*
CAS	RTPRG	EQYFGPGTRL	(BJ2S7)	7/23	30
CAS	RDPSGSH	EQYFGPG	(BJ2S7)	3/23	13
CAS	SRQQGFSS	YEQYFGP	(BJ2S7)	2/23	8.7
CASS	GQNRGS	SYEQYFG	(BJ2S7)	2/23	8.7
CA	GGLFRGSP	NEQFFGP	(BJ2S1)	1/23	4.3
CASS	SPFWG	YEQYFGPGTR	(BJ2S7)	1/23	4.3
CASS	EGAGED	YEQYFGP	(BJ2S7)	1/23	4.3
CAS	ELAGP	YEQYFGPGTR	(BJ2S7)	1/23	4.3
CAS	YSVRI	ETQYFGPGTR	(BJ2S5)	1/23	4.3
CA	TGLY	TGELFFGEGS	(BJ2S2)	1/23	4.3
CAS	KPGLAS	DTQYFGP	(BJ2S3)	1/23	4.3
CASS	EAHTSGG	TDTQYFG	(BJ2S3)	1/23	4.3
C	VAHSGSS	SGANVLT	(BJ2S6)	1/23	4.3

T cells in vivo infiltrating in synovium

BV	*NDN*	*Bj*		*Frequency*	*(%)*
CAS	SLG	TGELFFGEG	(BJ2S2)	19/27	70
CAS	TDGGPY	YGYTFGS	(BJ1S2)	2/27	7.0
CASSY	EAT	ETQYFGPGTR	(BJ2S5)	2/27	7.0
CAS	SLGLAGA	GELFFGE	(BJ2S2)	1/27	4.0
CASSY	SGTSGSR	DTQY	(BJ2S3)	1/27	4.0
CASSY	SRGL	DTQYFGPGTR	(BJ2S3)	1/27	4.0
CASS	ELRGVG	TQYFGPG	(BJ2S5)	1/27	4.0

*B Identical sequences between T cell clones induced by stimulation with purified CII in vitro and synovial T cells in vivo**

Patient	*BV*		*NDN*	*Bj*	
1	(BV13S1)	CAS	SLG	TGELF	(BJ2S2)
	(BV12)	CAI	TAEE	EQYFG	(BJ2S7)
2	(BV1)	CASS	VGRASG	YN	(BJ2S1)
3	(BV16)	CASS	RSSGSR	YE	(BJ2S7)

*Nucleotides were extracted from the band areas of SSCP gels indicated by arrows in figure 1.

BV13S1-SLG-BJ2S2 that expanded in the synovium recognised hCII, as shown by not only the SSCP analysis but also by its frequency as determined by the sequence analysis.

We also analysed the nucleotide sequences of the remaining three TCR B genes that were thought to recognise hCII (fig 1, arrows) after extraction of the DNA from SSCP gels. However, no amino acid motif was detected in the CDR3 regions and no bias in their BV gene usage was noted among these clones (table 2B).

Discussion

To our knowledge, the exact antigen target(s) for clonally expanded T cells has not yet been identified in the synovium of RA. To identify such antigens, we focused in this study on CII and investigated whether CII recognising T cell clones were among those accumulated T cells in the synovium. Our results strongly suggested that CII is recognised by the clonally expanded T cells in the synovium of RA patients.

In our study, we used the RT-PCR-SSCP system to detect the antigen responding T cell clones that would be induced by in vitro antigenic stimulation. Although this method could detect antigen induced clones, these clones may not be antigen specific. In this regard, we previously analysed the antigen specificity of the induced clones using an established system of an antigen specific T cell response—that is, human T lymphotropic virus type I (HTLV-I) associated myelopathy/tropic spastic paraparesis (HAM/TSP).[13] As a result, T cell clones that were expanded after in vitro culture with HTLV-I $Tax_{11–19}$ antigen and then detected by the RT-PCR-SSCP were found to be HTLV-I $Tax_{11–19}$-specific cytotoxic T cells. Considering this fact, the T cell clones that were induced by purified CII stimulation in this study is likely, at least in part, to include CII specific ones that could be expanded by in vitro CII stimulation. Although it may be necessary to establish T cell lines of clones to confirm the antigen specificity, our RT-PCR-SSCP system would provide an alternative method to identify the existence of antigen responding, even if not antigen specific, T cell clones without in vitro manipulating.

What we have found in this study are the T cell clones with TCR CDR3 sequences compatible with recognition of peptide derived from CII. As we used chromatographically purified CII, CII is very likely to have induced T cell expansion in vitro. However, even such highly purified CII might be contaminated with very small amounts of other articular antigens,[14] immune reaction to the antigens cannot be excluded completely. This would be an inevitable problem of experiments using purified antigens.

The T cell clones, which had identical TCR CDR3 sequences to the purified CII induced clones, were detected in the synovium of three of the 15 tested RA patients. These clones were thought to recognise CII in the synovium. Although such clones were found to share only a small part of the expanded clones in the synovium, such low frequency is reasonable, as a similar frequency of antigen specific T cells has also been reported in other lesions. For example, the frequency of pathogen specific T cells ranges from 0.1 to 2.2% even at the pathogen

affected inflammatory site.[15] It is probable that the remaining T cell clones might recognise other antigens in the joint or infiltrate antigen non-specifically.

We could not detect the T cell clones that are likely to recognise CII in the remaining 12 patients (80%). The T cell response to CII to make clonal expansion was detected only in a minority of the patients in our system. In this regard, T cell proliferation against CII is thought to correlate with the production of anti-CII antibody.[16] Previous studies have shown that anti-CII antibody is present in only 30% of patients with early RA, and decrease during the course of the disease.[8 9] As the RA patients enrolled in this study were with longstanding disease, the frequency of positive anti CII antibody titre would be much lower than 30%. In fact, none of the patients were found to be positive for anti-CII antibody by ELISA in this study (table 1). Thus it was predicted that the T cells that respond to CII would be at low frequency. Nevertheless, the RT-PCR-SSCP system clarified the existence of the minor populations of T cell clones that expanded in response to CII at the disease sites. Considered together, these findings suggest that CII might contribute to triggering RA in the early stages of the disease rather than representing a universal driving factor in advanced RA. Further studies using synovial samples from recently onset active arthritis would clarify the contribution of CII as a triggering antigen of RA.

Interestingly, identical clones were detected in patient 2 in hCII stimulated PBMC, bCII stimulated PBMC and in the synovium (fig 1C). As the amino acid similarity in hCII and bCII is about 90%,[17] a common epitope between hCII and bCII may be recognised by the T cell clone.

In conclusion, we demonstrated that a proportion of clonally expanded T cells in RA synovial lesions would respond to hCII in the affected site. Our findings suggest that expansion of oligoclonal T cells in the RA joints is driven at least in part by intra-articular autoantigens, such as CII.

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